EXHIBIT 1

Use of "anti-oxidants" during chemotherapy and radiotherapy should be avoided

Use of Antioxidants During Chemotherapy and Radiotherapy Should Be Avoided

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& METHODS

The term "complementary and alternative methods" (CAM) refers to products and regimens that individuals may employ either to enhance wellness, relieve symptoms of disease and side effects of conventional treatments, or cure disease. CAM articles provide evidence-based information on promising complementary and alternative methods, and inform clinicians of methods that may harm patients.

Use of Antioxidants During Chemotherapy and Radiotherapy Should Be Avoided

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ABSTRACT Many patients being treated for cancer use dietary supplements, particularly antioxidants, in the hope of reducing the toxicity of chemotherapy and radiotherapy. Some researchers have claimed, furthermore, that antioxidants also increase the effectiveness of cytotoxic therapy and have explicitly recommended their use. However, mechanistic considerations suggest that antioxidants might reduce the effects of conventional cytotoxic therapies. Preclinical data are currently inconclusive and a limited number of clinical studies have not found any benefit. Clinicians should advise their patients against the use of antioxidant dietary supplements during chemotherapy or radiotherapy. Such caution should be seen as the standard approach for any unproven agent that may be harmful. (*CA Cancer J Clin 2005;55:319–321.*) © American Cancer Society, Inc., 2005.

INTRODUCTION

Practicing oncologists are commonly asked by their patients if there is anything they can do to reduce the toxic effects of chemotherapy and radiotherapy and, if possible, help fight their cancer. The topic of perhaps greatest interest is vitamins and other nutritional supplements. It is estimated from survey data that 50% of cancer patients use some kind of dietary supplementation.[1,2] Patients often understand in a general sense that supplements "help protect the body," and the mechanism for protection against chemotherapy and radiotherapy toxicity is well understood. Radiotherapy and many chemotherapy agents act by producing free radicals; some vitamins and supplements, including vitamins C and E, are antioxidants and bind to free radicals, preventing oxidative damage.

Preclinical Evidence

There are considerable in vitro and animal data showing that vitamin C and other antioxidants can protect cells against radiation and chemotherapy.[3–5] It seems likely that they would therefore reduce treatment-related toxicities and there are promising, although not unequivocal, data that this indeed is the case.[6–10] However, it also follows that antioxidants might protect cancer cells, thereby reducing the oncologic effectiveness of cytotoxic therapy. This is the reason why most oncologists discourage patients from using antioxidants during treatment.

Proponents of antioxidant therapy believe that this policy is mistaken and expressly recommend that antioxidants should be taken during chemotherapy and radiotherapy. They claim that the protective effects of antioxidants are selective for normal cells, such that they can reduce toxicities without compromising oncologic efficacy. It is also sometimes claimed that antioxidants are directly cytotoxic and/or can actually increase the effectiveness of cytotoxic treatments. These claims are based on a variety of laboratory studies. For example, in vitro studies have reported that vitamins A, C, and E, as well as carotenoids, can enhance the effectiveness of chemotherapy and radiotherapy.[11–15] Yet some laboratory data suggest that

antioxidants might compromise the efficacy of cytotoxics. For example, the mechanism by which oxidized dehydroascorbic acid universally enters cells via glucose transporters and accumulates inside the cells in its reduced state (ascorbic acid) has been well described.[16] Cancer cells have been shown to exhibit upregulation of these facilitative glucose transporters and hence take up more glucose and more vitamin C than their normal neighbors.[17] This would suggest that the protective effect of vitamin C might be even greater for tumors than for normal cells. It has been empirically demonstrated that cancer cells can become resistant to oxidative injury by treatment with vitamin C.[18] Similarly, although there are in vitro data suggesting a direct antitumor effect for vitamin C, some investigators have claimed that these effects depend on the culture medium used and hence are of questionable validity.[19]

Clinical Evidence

Even if the laboratory data were not conflicting and confusing, they would be insufficient to guide clinical practice. There is no need here to recount the reasons why it is inappropriate to administer an agent to a cancer patient on the basis of cell culture studies and why we require data from human clinical trials. But it is worth restating that the harmful effects of antioxidants might be important even if they were small: a reduction of only a few percentage points in the efficacy of chemotherapy might lead to hundreds or thousands of deaths every year. Human trials therefore need to be large. There has been no attempt to mount the kind of trial needed to guide clinical practice, in which many hundreds of patients are randomized to receive chemotherapy or radiotherapy with or without antioxidants. Nonetheless, the clinical trial literature does provide some interesting data.

The antioxidant perhaps most widely used for treating cancer is vitamin C. The possibility that this compound may be useful in the treatment of cancer was first raised by Cameron and Campbell in 1974.[20] Subsequently, Pauling and Cameron published research suggesting a survival benefit from vitamin C.[21] The use of historical controls and the methods of patient selection weaken the level of evidence provided by this study. Subsequently, two randomized double-blind trials were conducted comparing placebo to vitamin C in patients with advanced cancers.[22,23] Neither study was able to show any objective improvement in disease progression or survival over placebo. Indeed, there seems to be somewhat worse survival in the vitamin C group.

A study that more directly addresses the issue of antioxidant use concurrently with cytotoxics is that of Lesperance, et al.[24] In this trial, 541 patients with early stage breast cancer who were prescribed megadoses of combination vitamins, minerals, and other antioxidants concurrent with standard therapy were compared with 180 well-matched controls. Breast cancer–specific survival ($P = .16$) and disease-free survival ($P = 0.07$) showed a trend toward worse survival in antioxidant-treated patients. Although many confounding factors may explain these differences in survival, the data should concern any oncologist who has patients considering antioxidant therapy.

It should also be noted that several large prevention trials have reported clinical data showing no benefit for supplementation. In fact, there are reports that it may be detrimental. Two trials, the Alpha-Tocopherol Beta-Carotene Cancer Prevention Study (ATBCCPS)[25] and the Beta-Carotene and Retinol Efficacy Trial (CARET),[26] demonstrated an increased relative risk for developing lung cancer in the high-risk cohort receiving beta carotene supplementation. A meta-analysis of 14 randomized trials of antioxidant supplementation for the prevention of gastrointestinal cancers found no evidence that antioxidant supplements are effective. A subgroup analysis of higher quality trials suggested a small increase in mortality among people taking antioxidants compared with those in the placebo group.[27] In the HOPE-TOO (Heart Outcomes Prevention Evaluation—The Ongoing Outcomes) trial, participants randomized to take either 400 IU of vitamin E daily or a placebo did not differ significantly with regard to incidence of or mortality from cancer overall or cancers that previous studies suggested might be prevented by vitamin E (prostate, lung, oral, colorectal, breast, and melanoma). However, people on vitamin E were more likely to develop heart failure.[28]

In another recent study, vitamin E had no effect on the incidence of second primary head and neck tumors among survivors of Stage I or II head and neck cancer previously treated with radiotherapy.[24] Although these chemoprevention trials are not directly applicable to the question of antioxidant use during treatment for active cancer, they do demonstrate that even though there was a plausible mechanism for antioxidant effect, good laboratory data, and promising results from preliminary human studies, antioxidants were found to do more harm than good when tested in randomized trials.

Taken together with treatment studies, these trials illustrate the complexity and the contradictory nature of existing data. Further study is necessary to clarify the role of antioxidants.

CONCLUSION

Pending the publication of suitable trials, clinicians must be guided by existing data in the context of a fundamental principle of medicine, "Primum non nocere." There are reasons to believe that taking antioxidants concurrently with chemotherapy or radiotherapy might be harmful; therefore, patients should be advised against it. Contrasting evidence from extensive human studies is needed before patients are advised to take antioxidants during cytotoxic therapy.

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EXHIBIT 2

Physician recommendations against anti-oxidant while on chemotherapy

Antioxidants and Chemotherapy

There has been a large increase in the use of antioxidants by the general public. Many cancer patients are taking antioxidants during their cancer treatment. Patients may be unaware that antioxidants can interfere with other treatments and have toxic effects at any dosage level.

The most common antioxidants are vitamins A (including beta-carotene), B6, C, and E; minerals (including zinc and selenium); bioflavnoids; glutathione; and most herbal medicines. Recent studies, however, have shown that there my be possible interactions between dietary antioxidants and treatments such as chemo and radiation therapies. Most chemotherapy drugs produce "reactive oxygen species" to destroy cancer cells. Antioxidants may consume these free oxygen radicals (I.E. toxic substances) in the body to prevent or lessen the breakdown of cells. By taking a large amount of antioxidants during cancer treatment, you may experience fewer side effects. However, you may also make the treatment less effective.

It is recommended to avoid a large intake of antioxidants (i.e. above the recommended daily allowance) during chemotherapy. In addition, if you are taking extra vitamins A or E, stop taking them at least two days before chemotherapy. These vitamins stay in the body longer than other antioxidants vitamins. In general, you may restart taking antioxidants two days after chemotherapy. It is extremely important that your doctor or nurse are aware of any "therapies" you may want to use during your cancer treatment. This includes any over-the-counter drugs. Working together may prevent any harmful effects that these combinations may cause.

By Chin Liu, Pharm. D.
(Reprinted from N.E.W.S. Bites, published by the Karmanos Cancer Institute)

EXHIBIT 3

Cancer cells up-regulate or concentrate "anti-oxidants" to protect themselves

1: Perez-Cruz I, Cárcamo JM, Golde DW.

Caspase-8 dependent TRAIL-induced apoptosis in cancer cell lines is inhibited by vitamin C and catalase.
Apoptosis. 2007 Jan;12(1):225-34.
PMID: 17031493 [PubMed - indexed for MEDLINE]

2: KC S, Cárcamo JM, Golde DW.

Vitamin C enters mitochondria via facilitative glucose transporter 1 (Glut1) and confers mitochondrial protection against oxidative injury.
FASEB J. 2005 Oct;19(12):1657-67.
PMID: 16195374 [PubMed - indexed for MEDLINE]

3: KC S, Cárcamo JM, Golde DW.

Antioxidants prevent oxidative DNA damage and cellular transformation elicited by the over-expression of c-MYC.
Mutat Res. 2006 Jan 29;593(1-2):64-79. Epub 2005 Aug 8.
PMID: 16085125 [PubMed - indexed for MEDLINE]

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Hypoxia-reoxygenation-induced mitochondrial damage and apoptosis in human endothelial cells are inhibited by vitamin C.
Free Radic Biol Med. 2005 May 15;38(10):1311-22.
PMID: 15855049 [PubMed - indexed for MEDLINE]

5: Karaguyvas N, Cárcamo JM, Stratis G, Golde DW.

Vitamin C protects HL60 and U266 cells from arsenic toxicity.
Blood. 2005 May 15;105(10):4004-12. Epub 2005 Jan 27.
PMID: 15677571 [PubMed - indexed for MEDLINE]

Vitamin C protects HL60 and U266 cells from arsenic toxicity.

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Although there is no compelling evidence that vitamin C has antitumor activity in humans, clinical trials are testing the hypothesis that ascorbic acid (AA) will enhance the efficacy of arsenic trioxide (As2O3) in myeloma. In vitro, AA cytotoxicity depends on its interaction with free transition metal ions in culture media leading to the generation of H2O2 and other reactive oxygen species (ROSs). Therefore, to circumvent the extracellular in vitro pro-oxidant effects of AA, we loaded HL60, U266, and RPMI-8226 cells with vitamin C by incubation with dehydroascorbic acid (DHA). Loading cells in this manner resulted in prominent, dose-dependent protection of As2O3-treated cells as measured by viability, colony formation, and apoptosis assays. Glutathione depletion enhanced cell sensitivity to the cytotoxic effects of As2O3 and vitamin C loading provided protection. AA was found to generate cytotoxic concentrations of H2O2 in culture medium without cells and copper/iron chelators inhibited this reaction. However, AA did not generate H2O2 in simple buffer or human plasma. Direct incubation with AA resulted in increased intracellular ROSs, whereas DHA incubation decreased it. These results clarify an apparent paradox and indicate that vitamin C loading in HL60, U266, and RPMI-8226 cells ameliorates As2O3 cytotoxicity.

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Chemotherapy induces an increase in coenzyme Q10 levels in cancer cell lines.
Free Radic Biol Med. 2006 Apr 15;40(8):1293-302. Epub 2005 Dec 13.
PMID: 16631519 [PubMed - indexed for MEDLINE]

2: Gstrein C, Lopez-Jones M, Goldberg GL, Romney SL, Klimpel D.

Coenzyme Q10 and lipid-related gene induction in HeLa cells.
Am J Obstet Gynecol. 2004 May;190(5):1432-4.
PMID: 15167860 [PubMed - indexed for MEDLINE]

Chemotherapy induces an increase in coenzyme Q10 levels in cancer cell lines.

Brea-Calvo G, Rodríguez-Hernández A, Fernández-Ayala DJ, Navas P, Sánchez-Alcázar JA.

Centro Andaluz de Biología del Desarrollo, Universidad Pablo de Olavide, Carretera de Utrera Km 1, Sevilla, Spain.

Free radicals have been implicated in the action of many chemotherapeutic drugs. Here we tested the hypothesis that camptothecin and other chemotherapeutic drugs, such as etoposide, doxorubicin, and methotrexate, induce an increase in coenzyme Q(10) levels as part of the antioxidant defense against free radical production under these anticancer treatments in cancer cell lines. Chemotherapy treatment induced both free radical production and an increase in coenzyme Q(10) levels in all the cancer cell lines tested. Reduced coenzyme Q(10) form levels were particularly enhanced. Coenzyme Q(10)-increased levels were associated with up-regulation of COQ genes expression, involved in coenzyme Q(10) biosynthesis. At the translational level, COQ7 protein expression levels were also increased. Furthermore, coenzyme Q(10) biosynthesis inhibition blocked camptothecin-induced coenzyme Q(10) increase, and enhanced camptothecin cytotoxicity. Our findings suggest that coenzyme Q(10) increase is implicated in the cellular defense under chemotherapy treatment and may contribute to cell survival.

PMID: 16631519 [PubMed - indexed for MEDLINE]

EXHIBIT 4

"anti-oxidants" promote cancer

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Beta-carotene in multivitamins and the possible risk of lung cancer among smokers versus former smokers: a meta-analysis and evaluation of national brands.
Cancer. 2008 Apr 21. [Epub ahead of print]
PMID: 18429004 [PubMed - as supplied by publisher]

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Alpha-Tocopherol and beta-carotene supplements and lung cancer incidence in the alpha-tocopherol, beta-carotene cancer prevention study: effects of base-line characteristics and study compliance.
J Natl Cancer Inst. 1996 Nov 6;88(21):1560-70.
PMID: 8901854 [PubMed - indexed for MEDLINE]

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J Natl Cancer Inst. 1996 Nov 6;88(21):1550-9.
PMID: 8901853 [PubMed - indexed for MEDLINE]

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The Beta-Carotene and Retinol Efficacy Trial: incidence of lung cancer and cardiovascular disease mortality during 6-year follow-up after stopping beta-carotene and retinol supplements.
J Natl Cancer Inst. 2004 Dec 1;96(23):1743-50.
PMID: 15572756 [PubMed - indexed for MEDLINE]

EXHIBIT 5

Cytoskeleton disruptors as cancer therapeutics (but toxic)

Although, this literature search was done for a single cytoskeleton disruptor, it shows that they are wide-spectrum drugs, applied for all cancers. It only covers one decade from mid 1960 to mid 1970. The literature on this topic is extensive and includes over 100,000 scientific reports.

To access the links below input them in the address bar of the browser and you would be taken directly to the PubMed database at the National Library of Medicine.

General mechanism (cytoskeletal binding):
http://www.ncbi.nlm.nih.gov/pubmed/1260766?ordinalpos=285&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

http://www.ncbi.nlm.nih.gov/pubmed/5656643?ordinalpos=21343&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

http://www.ncbi.nlm.nih.gov/pubmed/5026289?ordinalpos=20690&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

Early clinical studies
http://www.ncbi.nlm.nih.gov/pubmed/14023264?ordinalpos=21665&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

http://www.ncbi.nlm.nih.gov/pubmed/13992692?ordinalpos=21661&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

http://www.ncbi.nlm.nih.gov/pubmed/14051497?ordinalpos=21658&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

http://www.ncbi.nlm.nih.gov/pubmed/14018556?ordinalpos=21659&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

http://www.ncbi.nlm.nih.gov/pubmed/14226130?ordinalpos=21606&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

Established more than 50 years ago as therapy for many malignancies:

CHILDHOOD LEUKEMIA
http://www.ncbi.nlm.nih.gov/pubmed/14126715?ordinalpos=21663&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

ADVANCED BREAST CANCER
http://www.ncbi.nlm.nih.gov/pubmed/14116216?ordinalpos=21642&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

SEVERAL ADULT SOLID TUMORS
http://www.ncbi.nlm.nih.gov/pubmed/14116215?ordinalpos=21643&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

http://www.ncbi.nlm.nih.gov/pubmed/14088830?ordinalpos=21652&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

GLIOMAS
http://www.ncbi.nlm.nih.gov/pubmed/14078103?ordinalpos=21646&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

LYMPHOMA AND LEUKEMIA
http://www.ncbi.nlm.nih.gov/pubmed/14063417?ordinalpos=21647&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

http://www.ncbi.nlm.nih.gov/pubmed/13968454?ordinalpos=21660&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

METASTATIC WILM'S TUMOR (KIDNEY) IN CHILDREN
http://www.ncbi.nlm.nih.gov/pubmed/14075630?ordinalpos=21650&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

MEDULLOBLASTOMA WITH METASTASES
http://www.ncbi.nlm.nih.gov/pubmed/14057683?ordinalpos=21651&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

Hodgkin's disease, reticulum cell sarcoma, lymphosarcoma, carcinoma of the breast, acute leukemia and choriocarcinoma
http://www.ncbi.nlm.nih.gov/pubmed/14158543?ordinalpos=21622&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

NEUROBLASTOMA AND NEPHROBLASTOMA
http://www.ncbi.nlm.nih.gov/pubmed/14129328?ordinalpos=21636&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

GYNECOLOGICAL CANCERS
http://www.ncbi.nlm.nih.gov/pubmed/14209555?ordinalpos=21609&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

REFRACTORY RETICULUM CELL SARCOMA
http://www.ncbi.nlm.nih.gov/pubmed/5329865?ordinalpos=21522&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

METASTATIC GERMINAL TUMORS
http://www.ncbi.nlm.nih.gov/pubmed/6006141?ordinalpos=21506&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

TESTICULAR TUMORS
http://www.ncbi.nlm.nih.gov/pubmed/5925278?ordinalpos=21512&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

SOFT TISSUE SARCOMA
http://www.ncbi.nlm.nih.gov
/pubmed/5950594?ordinalpos=21517&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_
ResultsPanel.Pubmed_RVDocSum

EMBRYONAL RHABODOMYOSARCOMA
http://www.ncbi.nlm.nih.gov/pubmed/6020189?ordinalpos=21488&itool=EntrezSystem2.
PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

EWING'S SARCOMA
http://www.ncbi.nlm.nih.gov/pubmed/4163333?ordinalpos=21489&itool=EntrezSystem2.
PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

CHORDOMA OF THE SPINE
http://www.ncbi.nlm.nih.gov/pubmed/4528471?ordinalpos=6951&itool=EntrezSystem2.P
Entrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

MULTIPLE MYELOMA
http://www.ncbi.nlm.nih.gov/pubmed/4812771?ordinalpos=6939&itool=EntrezSystem2.P
Entrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

OSTEOSARCOMA
http://www.ncbi.nlm.nih.gov/pubmed/804824?ordinalpos=4381&itool=EntrezSystem2.PE
ntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

SQUAMOUS CELL CARCINOMA
http://www.ncbi.nlm.nih.gov/pubmed/4713171?ordinalpos=5795&itool=EntrezSystem2.P
Entrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

MESOTHELIOMA
http://www.ncbi.nlm.nih.gov/pubmed/1260651?ordinalpos=3176&itool=EntrezSystem2
.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

CARCINOMA OF THE STOMACH
http://www.ncbi.nlm.nih.gov/pubmed/186174?ordinalpos=3141&itool=EntrezSystem2.PE
ntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

THYMUS
http://www.ncbi.nlm.nih.gov/pubmed/181132?ordinalpos=3160&itool=EntrezSystem2.PE
ntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

ADENOCARCINOMA OF THE SMALL INTESTINE
http://www.ncbi.nlm.nih.gov/pubmed/900333?ordinalpos=2978&itool=EntrezSystem2.PE
ntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

ENDODERMAL GERM CELL CARCINOMA
http://www.ncbi.nlm.nih.gov/pubmed/479268?ordinalpos=2826&itool=EntrezSystem2.PE
ntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

ANAPLASTIC CARCINOMA

http://www.ncbi.nlm.nih.gov/pubmed/7448439?ordinalpos=2783&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

MIXED MULLERIAN TUMOR OF THE FALLOPIAN TUBE
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ALIMENTARY TRACT MALIGNOMAS
http://www.ncbi.nlm.nih.gov/pubmed/6212524?ordinalpos=2655&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

malignant fibrous histiocytoma of bone
http://www.ncbi.nlm.nih.gov/pubmed/2988736?ordinalpos=1990&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

AFRICAN LYMPHOMA
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DISSEMINATED MELANOMA
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LEUKEMIA AND BURKITT'S TUMOR
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LEIOMYOSARCOMA
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RETINOBLASTOMA
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CENTRAL NERVOUS SYSTEM TUMORS AND OTHER MALIGNANCIES
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HEPATOBLASTOMA AND HEPATOMA (LIVER)
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PANCREATIC CARCINOMA IN CHILDREN
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GANGLIONIC SARCOMA
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CONGENITAL LETTERER-SIWE DISEASE
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STOMATOLOGIC CANCERS
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ANGIOSARCOMA
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HEMANGIOPERICYTOMA
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PULMONARY CARCINOMA
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OVARIAN MALIGNANCIES
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KAPOSI'S SARCOMA
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GENERALIZED MALIGNANT SCHWANNOMA
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UROLOGICAL TUMORS
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BRONCHIAL CANCERS
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OSTEOGENIC SARCOMA
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BLADDER AND PROSTATE
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METASTATIC COLORECTAL LIVER CARCINOMA
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GESTATIONAL CHORIOCARCINOMA
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NON-HODGKINS LYMPHOMA
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SMALL CELL CARCINOMA OF THE LUNG
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LUNG CANCER
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GASTROINTESTINAL TUMORS
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ESTHESIONEUROBLASTOMA
http://www.ncbi.nlm.nih.gov/pubmed/4711625?ordinalpos=20392&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

Single study showing responses in advanced lymphosarcoma, reticulum cell sarcoma, Hodgkin's disease, breast cancer, bladder cancer, and carcinomas of unknown primary site; carcinoma of lung, melanoma, carcinoma of head and neck, cervix, prostate, pancreas, neuroblastoma and rectum.
http://www.ncbi.nlm.nih.gov/pubmed/4352365?ordinalpos=20376&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum

EXHIBIT 6

Epidermal Growth Factor to illustrate the redundancy of growth pathways

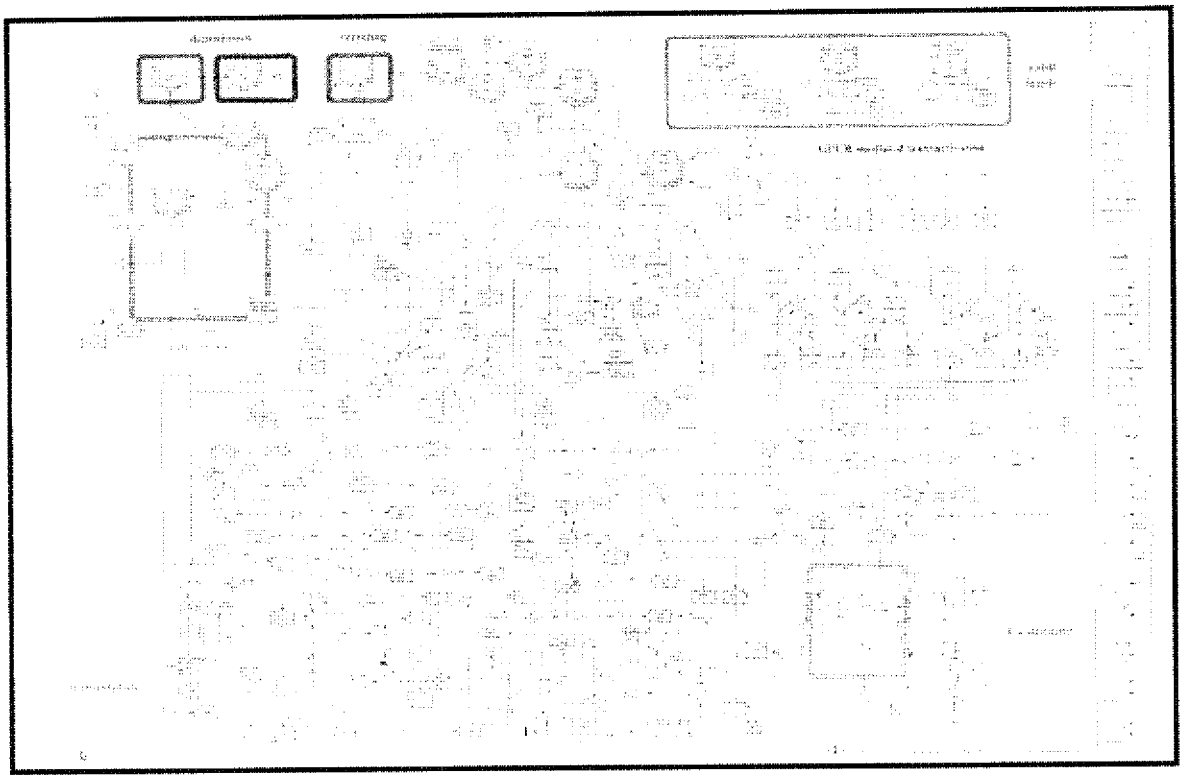

EXHIBIT 7

Oleuropein is non-toxic

Petkov V, Manolov P.

Pharmacological analysis of the iridoid oleuropein.
Arzneimittelforschung. 1972 Sep;22(9):1476-86. No abstract available.
PMID: 4678608 [PubMed - indexed for MEDLINE]

EXHIBIT 8

Oleuropein as a non-toxic cytoskeleton disruptor

Application No: 10/712,423, Hamdi K Hamdi and Raquel Castellon

Exhibit 8 continued...

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Oleuropein, a non-toxic olive iridoid, is an anti-tumor agent and cytoskeleton disruptor

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Abstract

Oleuropein, a non-toxic secoiridoid derived from the olive tree, is a powerful antioxidant and anti-angiogenic agent. Here, we show it to be a potent anti-cancer compound, directly disrupting actin filaments in cells and in a cell-free assay. Oleuropein inhibited the proliferation and migration of advanced-grade human tumor cell lines in a dose-responsive manner. In a novel tube-disruption assay, Oleuropein irreversibly rounded cancer cells, preventing their replication, motility, and invasiveness; these effects were reversible in normal cells. When administered orally to mice that developed spontaneous tumors, Oleuropein completely regressed tumors in 9–12 days. When tumors were resected prior to complete regression, they lacked cohesiveness and had a crumbly consistency. No viable cells could be recovered from these tumors. These observations elevate Oleuropein from a non-toxic antioxidant into a potent anti-tumor agent with direct effects against tumor cells. Our data may also explain the cancer-protective effects of the olive-rich Mediterranean diet.

Keywords: Mediterranean diet; Actin; Matrigel; Polyphenol; Elenolic acid; Hydroxytyrosol; Chemotherapy; Oncology; Cancer; Chemoprevention

Large epidemiological studies encompassing several countries showed that the Mediterranean region had a significantly reduced all-cause death rate [1–5]. This reduction was mainly due to decreased incidence of cardiovascular disease and cancer. The protective effect was attributed to the Mediterranean diet, rich in fruits and vegetables, whole grains, and olive products [5]. The Lyon Heart Study, a large randomized clinical trial, demonstrated that a modified diet based on that of the Mediterranean reduced total deaths by 56% and decreased the cancer risk by 61% at a 4-year follow up [1]. Other diets, similarly rich in fruits and vegetables but lacking the olive component, were not equally protective [3,4,6,7]. Evidence for the protective role of olives was also derived by comparing olive oil consumption in the Mediterranean countries to that of the United States. Mediterranean populations consume 20 times more olive oil than Americans; correspondingly, their cancer risk is at least half [3]. More direct evidence for the protective role of olive oil against cancer has been recently published by Filik and Ozyilkan [8].

Olive oil is rich in oleic acid and other monounsaturated fats with various biological actions. In recent studies, diets containing 15% olive oil significantly reduced chemically induced pre-cancerous lesions in rat breast and colon [9–11]. Even though the authors anticipated that a similar proportion of soy oil would be equally protective, this did not occur, suggesting that the chemo-preventive capacity of olive oil was not due to unsaturated fatty acids. A similar study determined that a high corn oil diet allowed the development of malignant adenocarcinomas in rats, whereas a high olive oil diet did not [12]. In addition to fatty acids, olives and olive oil are rich in powerful antioxidants such as polyphenols and flavonoids with diverse biological activities [13,14].

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Numerous lines of evidence demonstrate that antioxidants protect against DNA damage, a major step in oncogenic processes [15]. Oleuropein is the most abundant of the phenolic compounds in olives [16]. It actively scavenges reactive oxygen [17] and nitrogen species [18] as well inducing the production of nitric oxide in macrophages [19]. In addition, we have previously shown that Oleuropein is anti-angiogenic [20]. Even though the above qualities advance it as a major candidate for explaining the protective role of the Mediterranean diet, in this paper we present further evidence that Oleuropein has direct anti-tumor effects.

Materials and methods

Cell culture. Normal human skin fibroblasts (NL-Fib) and the following human advanced-grade tumor cell lines were purchased from the American Type Culture Collection, ATCC (Manassas, VA) and cultured according to the supplier's instructions: LN-18, poorly differentiated glioblastoma; TF-1a, erythroleukemia; 786-O, renal cell adenocarcinoma; T-47D, infiltrating ductal carcinoma of the breast-pleural effusion; MCF-7, mammary gland adenocarcinoma, pleural effusion; RPMI-7951, malignant melanoma of the skin-lymph node metastasis; and LoVo, colorectal adenocarcinoma-supraclavicular region metastasis. All cells were used within 10 passages after arrival. Cell culture reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA). Cell culture flasks and sterile pipettes were obtained from USA Scientific (Ocala, FL). Nalgene sterile filtration units were from Fisher Scientific (Hampton, NH). All cultures were maintained in a 37 °C, 5% CO_2 atmosphere.

Cell proliferation. Approximately 5×10^3 cells (TF-1a; 786-O, T-47D, RPMI-7951, LoVo, and NL-Fib) were seeded in triplicate wells of 96-well plates in 100 μl of their respective growth medium with increasing doses of purified Oleuropein (Indofine Chemical, Hillsborough, NJ). Cell proliferation was determined on day 5 using the CellTiter MTS cell proliferation kit (Promega, Madison, WI) according to the manufacturer's instructions. Absorbance at 490 nm was measured with a Bio-Tek Instruments microplate reader. Background readings (medium ± Oleuropein without cells + CellTiter reagents) were subtracted from the experimental readings.

β-Glucosidase treatment. Five hundred microliters of a 1% solution of Oleuropein in fibroblast tissue culture medium was incubated with ±25U of purified β-glucosidase from almonds (Sigma Chemical, St. Louis, MO) for 2 days at 37 °C. Control and enzymatically treated Oleuropein was then diluted to a final concentration of 0.025% for the cell proliferation assay as described above.

Cell migration. Fibroblasts and adherent tumor cell lines (LN-18, RPMI-7951, 786-O, and T-47D) were grown to confluence in 24-well tissue culture plates. The monolayer was wounded vertically using the wooden end of a sterile cotton swab stick. For more extensive damage, monolayers were also wounded in the shape of a cross with a sterile 1 ml pipette tip. Detached cells were removed by washing with phosphate-buffered saline (PBS) and the remaining monolayer was covered with 500 μl of growth medium with or without 0.01% Oleuropein. Original edges were marked for reference. Alternatively, some experiments measuring radial migration were carried out as follows: approximately 1×10^5 cells in a 5 μl volume were deposited as a drop in the center of a 24-well plate and incubated for 4–6 h at 37 °C to allow for adherence. The wells were subsequently washed with PBS to remove free-floating cells and covered with 500 μl of growth medium with or without 0.01% Oleuropein. In all cases, cell migration was monitored daily by phase-contrast microscopy (Swift Instruments International). At the conclusion of the experiment, cells were washed with PBS, fixed with 100% methanol at room temperature for 15 min, stained with Mayer's hematoxylin (Sigma) for 5 min, and rinsed with distilled water prior to photography with a Kodak MDS100 digital camera.

Reversibility of cell rounding. Fibroblasts and adherent tumor cells were seeded at approximately 5×10^4/well in a 24-well plate. Oleuropein was added to their respective growth medium to a final concentration of 0.01% followed by 24-h incubation. The cells were carefully washed at least five times with 2 ml of growth medium per wash and the medium was replaced with normal growth medium. Cells were photographed 48 h later.

Cell invasion. Falcon cell culture inserts (3 μm pore size) were cooled to −20 °C on top of their corresponding 24-well plates (Becton–Dickinson Discovery Labware, Bedford, MA) and overlaid with a 3-mm layer of ice-cold Matrigel (Becton–Dickinson) according to the manufacturer's instructions. Matrigel solidification occurred at 37 °C for at least 1 h. Subsequently, 100 μl of 0.5% fetal calf serum (FCS)-containing culture medium with approximately 2×10^5 LoVo colorectal cancer cells was added to the insert, whereas normal culture medium containing 10% FCS was added to the bottom well as a chemo-attractant. After a 4- to 6-h incubation, 11 μl of a 1% Oleuropein solution was added to the top chamber of duplicate wells. Cells were observed daily by phase-contrast microscopy and photographed. On day 10, the filters were removed from the cell culture inserts, fixed in methanol, and stained with Mayer's hematoxylin.

Tube-disruption assay. Triplicate wells of a previously frozen 96-well tissue culture plate were coated with 50 μl of Matrigel. After solidification at 37 °C, approximately 1×10^5 RPMI-7951 melanoma cells were added in 100 μl of 0.5% FCS-containing culture medium. After a 24-h incubation to allow for the formation of vascular-like tubes, 11 μl of a 1% Oleuropein solution was added. Some Oleuropein-treated wells also received D-glucose (Sigma) to a final concentration of 3.5% (weight/volume). Cells were observed hourly by phase-contrast microscopy and photographed as described above.

Immunofluorescence. Approximately 1×10^3 breast cancer cells (MCF-7) were seeded in a TiterTek II culture chamber slide (USA Scientific) and allowed to adhere for 24 h at 37 °C. The culture medium was changed to 0.5% FCS-containing medium with or without 0.01% Oleuropein. Some Oleuropein-treated wells had been pre-incubated in 0.5% FCS medium containing 3.5% D-glucose for 5 min. After a 2-h incubation, the slide was washed with PBS, fixed in ice-cold methanol for 15 min, and allowed to dry. After rehydration in PBS, the slide was blocked with 5% normal rabbit serum in PBS and processed for immunofluorescence as previously described [21]. Secondary antibody was a rabbit anti-mouse conjugated to TRITC (Sigma), 1:1000 in PBS, whereas the primary antibody was a mouse monoclonal against pan-actin (LabVision; Freemont, CA) at 2 μg/ml in PBS. To determine non-specific staining, we used the irrelevant FITC-rabbit anti-goat IgG at 1:1000 (Sigma). Final images were derived from merging the FITC and TRITC-filtered images using Adobe Photoshop software.

Purified actin filament disruption. This assay was a modification of the actin polymerization kit protocol (Cytoskeleton; Denver, CO). All solutions were prepared according to the manufacturer's instructions. G-actin was incubated for 1 h at RT with "G" buffer ± 0.01% solution of Oleuropein in "G" buffer. For visualization, 5 μl of G-actin solution ± Oleuropein was deposited on a clean, poly-lysine-coated glass slide together with 1 μl TRITC-phalloidin (Sigma) dissolved in methanol according to the manufacturer's instructions. After the addition of 5 μl of actin polymerization buffer, slides were incubated for 1 min at RT, cover-slipped, and photographed under a fluorescence microscope.

Animal studies. Three to four mice of the same gender were housed in individual cages with food and water ad libitum. They were in a temperature- and humidity-controlled environment with filtered air and regular light/dark cycles. A proprietary, house-established inbred strain of Swiss albino mice that spontaneously develops soft tissue sarcomas was used (H_2RC, Orange, CA). This mouse strain was

developed by inbreeding six generations arising from the F1 progeny of a male founder mouse that developed a spontaneous tumor without human intervention. Approximately 25% of male and female mice developed tumors around 1 year of age. If necessary to improve tumor visualization, hair was removed from the tumor area using a depilatory cream. After the tumors reached a visible diameter of at least 2 cm, mice were divided into two groups: five were assigned to the control (untreated) group, whereas 15 were assigned to the treatment group. Fourteen mice received 1% Oleuropein in their drinking water, which they consumed ad libitum. Pilot experiments had already shown that this was the maximum concentration of Oleuropein that could be used, since higher concentrations were too bitter and the mice refused to drink. Tumor size was monitored daily. On day 5, one of the Oleuropein-treated mice had its tumors removed for examination and development of primary tumor cell lines. The remaining animals in the treatment group continued to drink the Oleuropein-containing water until their tumors completely regressed, usually in 9–12 days. Untreated mice exhibited 100% mortality by the 10th day, whereas all 12 Oleuropein-treated mice remain(ed) tumor-free for the remainder of their lifespan. One mouse was given a 500 µl intraperitoneal injection of a 10% Oleuropein solution in Hanks' phosphate-buffered saline every 12 h for 2 days. On the third day, the tumor was resected, examined, and photographed.

Statistical analysis. Paired Student's *t* test was conducted using GraphPad Prism 4 software.

Results

Cell proliferation

The effect of Oleuropein on cell proliferation was assessed on normal human fibroblasts and tumor cell lines derived from advanced-grade human tumors (TF-1a; 786-O, T-47D, RPMI-7951, and LoVo). Cells were incubated with increasing doses of Oleuropein (0.005–0.025%). After 5 days, cell numbers were measured using a tetrazolium salt-based assay (Fig. 1). Optical densities are shown rather than normalized values to demonstrate the differential growth rates of the cell lines with respect to one another. All cell lines were growth-inhibited in a dose-dependent manner by the addition of Oleuropein. However, in the case of 786-O (renal cell adenocarcinoma) and normal fibroblasts, only the highest concentration was effective.

β-Glucosidase treatment of Oleuropein

In order to investigate whether the glucose moiety in Oleuropein affected its biological activity, we removed it with β-glucosidase treatment for 2 days prior to its use in the cell proliferation assay as above. When normal fibroblasts were counted on day 5, β-glucosidase-treated Oleuropein was less effective in inhibiting cell proliferation than the non-treated control (Fig. 2).

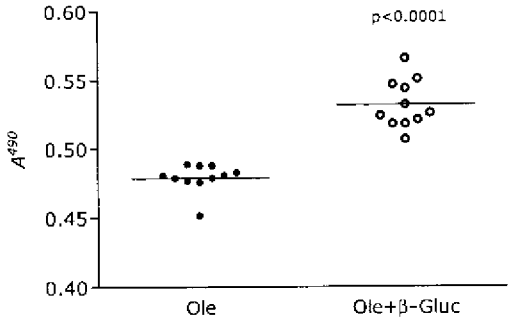

Fig. 2. β-Glucosidase decreases anti-proliferative activity of Oleuropein. Oleuropein was incubated with ±β-glucosidase for 48 h at 37 °C and added to approx. 5×10^3 normal fibroblasts. Cell proliferation was measured on day 5 with the CellTiter kit. Bars indicate mean of absorbance readings from four experiments.

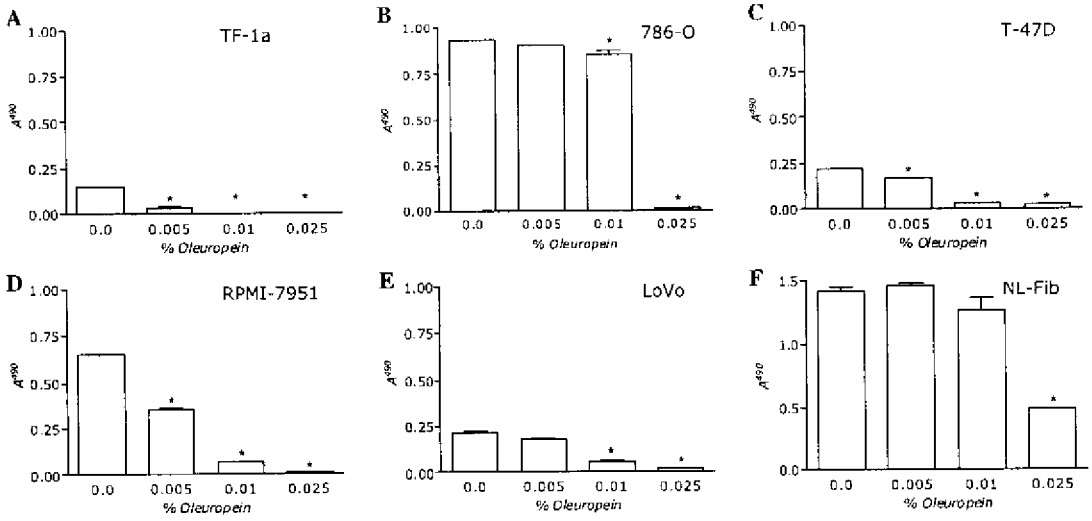

Fig. 1. Oleuropein inhibits cell proliferation. Approximately 5×10^3 human tumor cells (A–E) or normal fibroblasts (F) were seeded in 96-well plates with increasing doses of Oleuropein. Cell proliferation was measured on day 5 with the CellTiter kit. Bars indicate means ± standard deviation of absorbance readings from two experiments in triplicate. $^*p < 0.05$ vs. control.

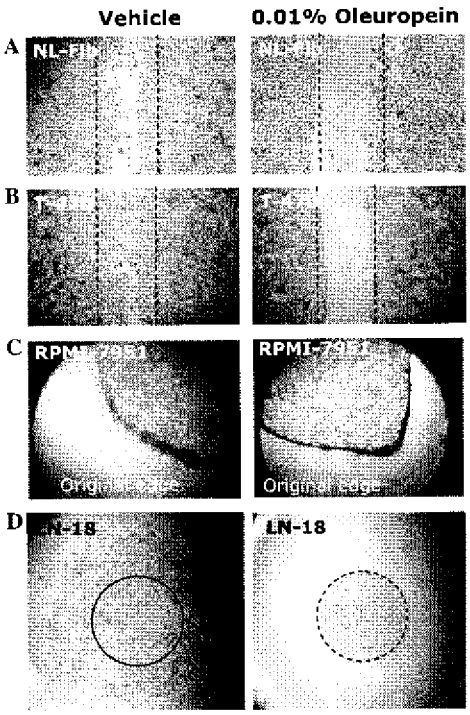

Fig. 3. Oleuropein inhibits cell migration. Confluent monolayers were wounded vertically (standard wound assay, A and B) or using a cross pattern (C). Alternatively, cells were seeded in one spot (radial migration assay, D). In all cases, detached cells were washed away and Oleuropein was added to 0.01% in fresh culture medium. Cells were fixed and stained on day 3. Dotted lines mark original edges. Magnification: 100× (A,B); 40× (C); and 20× (D).

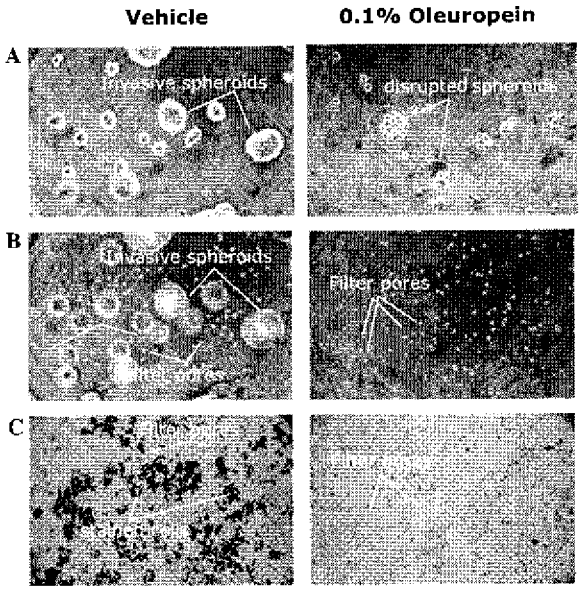

Fig. 4. Oleuropein inhibits tumor cell invasion. LoVo colorectal carcinoma cells in 0.5% serum medium ± Oleuropein were plated inside transwell inserts coated with undiluted Matrigel. The bottom culture chamber contained 10% serum medium as a chemo-attractant. (A) The migrating tumor cell spheroids on day 5, with the camera focusing on the cells. (B) The same field as (A) with the camera focusing on the filter. On day 10, filters were removed, fixed, and stained. (C) The stained filters at the conclusion of the experiment. Magnification: 100×.

Cell migration

Using three variations of cell migration assays, we show that 0.01% Oleuropein completely inhibits cell motility in all cell lines. Representative photographs are shown in Fig. 3. Panels A and B show the inhibition of normal fibroblasts and T-47D breast cancer cell migration by Oleuropein using the traditional monolayer wound assay. To better define the wound edge from the migrating cells, we modified this assay and increased the severity of the wound by making a cross pattern; this allowed the edge of the monolayer to curl, making it more visible. Using RPMI-7951 melanoma cells, we clearly show their migratory inhibition by Oleuropein (Fig. 3C). In panel D, we demonstrate a similar effect of Oleuropein on the radial migration of LN-18 glioblastoma cells.

Cell invasion

Using the highly invasive, spheroid-forming colon carcinoma cell line LoVo, we found that 0.1% Oleuropein completely blocked the invasion of tumor cells through a thick, undiluted Matrigel layer to the other side of the filter membrane (Fig. 4C). During this experiment, we noticed that Oleuropein-treated spheroids were disrupted and were incapable of moving through the matrix (Figs. 4A and B). By focusing on the filter, it is clear that the tumor spheroids in the Oleuropein-treated wells are far from the membrane whereas in the control, they appear close by. Interestingly, Oleuropein-treated spheroids contained rounded cells and appeared less cohesive (Fig. 4A).

Tube disruption

To study the kinetics of cell rounding, we modified the existing tube-formation assay on Matrigel. Cancer cell invasion assays in Boyden chambers use soft Matrigel (1–2 mg of protein/ml); however, tube-formation assays use hard Matrigel (9–12 mg protein/ml) to provide node anchoring. The tube-formation assay is used to study pro/anti angiogenic effects of substances on vascular endothelial cells. This is possible because vascular endothelial cells seeded on hard Matrigel undergo a programmed series of morphological changes, including tube formation and retraction. Endothelial cell tube formation on hard Matrigel occurs by cell motility and cell–cell contact. The tubes, however, are not stable and retract into clumps within 24–48 h [22]. Taking advantage of the "vascular mimicry" behavior of melanoma cells [23], we show that the human melanoma cell

line RPMI-7951 can also form tubes on hard Matrigel (Fig. 5). Unlike the vascular endothelial cell tubes, melanoma tubes collapse by retraction in 1 week. In certain cases, they never retract and cells invade the matrix (not shown). When added to the tubing phase of the assay, 0.1% Oleuropein disrupted the tubes in situ by rounding the cells and preventing tube retraction (Fig. 5). This process is relatively fast, occurring within 2 h; this contrasts with tube retraction, which occurs within 1 week. Since Oleuropein has a glucose moiety, we explored the possibility that it could enter the cell through the glucose transport system. Interestingly, co-incubation with excess D-glucose decreased the ability of Oleuropein to induce cell rounding, suggesting this as a possible mechanism of entry.

Reversibility of cell rounding

We next investigated whether the Oleuropein-induced cell rounding was reversible by extensively washing the Oleuropein-treated melanoma tubes and subsequent culturing in normal growth medium. Even after thorough washing and incubation in normal medium for 10 days, tumor cells remained immobile, round, and did not proliferate (not shown). Because of the possibility that Matrigel could act as a sponge to sequester Oleuropein, we decided to repeat the experiment on Matrigel-free cultures of RPMI-7951 melanoma cells as well as normal cells. We found that 0.01% Oleuropein rounded both normal and tumor cells; however, only the normal cell rounding was reversible after washing. Within 48 h, normal cells flattened out and again became mobile (Fig. 6). On the contrary, cancer cells remained immobilized after washing even after 1 week of culture in normal growth medium (not shown).

Actin filament disruption

Given that Oleuropein-induced cell rounding occurred within 2 h, we hypothesized that Oleuropein could be affecting the cytoskeleton. Adherent breast carcinoma cells (MCF-7) grown on chamber slides were treated with 0.01% Oleuropein for 2 h, fixed, and stained with a pan-actin monoclonal antibody. As shown in Fig. 7A, Oleuropein treatment dramatically disrupts the organization of actin filaments within the cells. This disruption coincided with the 2-h time frame for cell rounding observed previously. Co-incubation with excess D-glucose decreased the ability of Oleuropein to disrupt the cytoskeleton, suggesting the involvement of the glucose transport system. Fig. 7B demonstrates that Oleuropein directly disrupted actin filaments in a cell-free assay. The nature and kinetics of the disruption is currently under investigation.

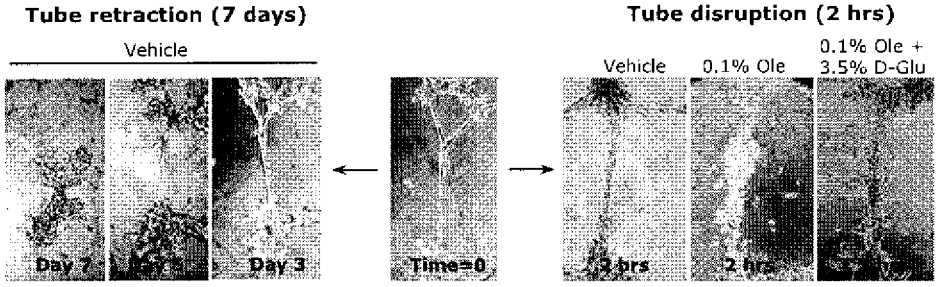

Fig. 5. Oleuropein disrupts melanoma tubes on Matrigel. RPMI-7951 human melanoma cells were seeded on undiluted Matrigel and allowed to form tubes. Left panels show the normal tube retraction over time. Right panels demonstrate the effect of Oleuropein (Ole) on tube structure. Addition of 0.1% Oleuropein induced cell rounding within 2 h. Excess D-glucose partially inhibited Oleuropein's disruptive action. Magnification: 100×.

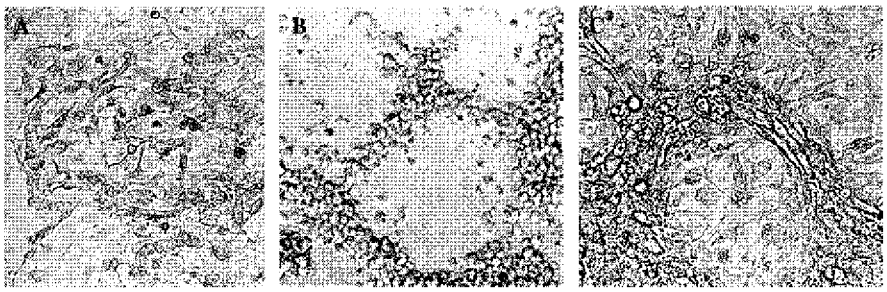

Fig. 6. Oleuropein-induced cell rounding is reversible in normal cells. Confluent monolayers of normal skin cells (A) were treated with 0.01% Oleuropein. After 2 h, the cells were photographed (B) and thoroughly washed. The same field was re-photographed after 48 h of incubation in normal growth medium (C). Magnification: 200×.

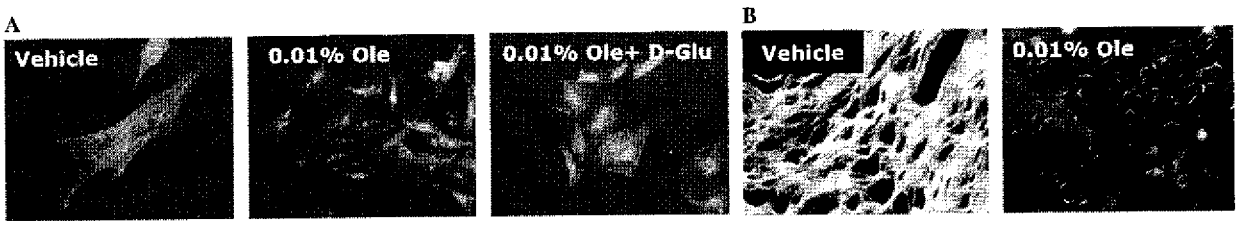

Fig. 7. Oleuropein disrupts actin filaments. (A) Immunofluorescence. Adherent MCF-7 cells were incubated for 2 h ± 0.01% Oleuropein (Ole) ± excess D-glucose (D-Glu). Actin filaments were stained with a pan-actin antibody and TRITC-anti-mouse IgG. (B) Purified actin. G-actin (pre-incubated for 1 h ± 0.01% Oleuropein) was polymerized on a glass slide. Filaments were visualized with TRITC-phalloidin. Magnification: 200×.

Animal studies

Having established Oleuropein's efficacy in vitro, we proceeded to determine its anti-tumor action in vivo. Using Swiss albino mice that spontaneously develop soft tissue sarcomas, we determined that 1% Oleuropein in the drinking water (consumed ad libitum) induced dramatic tumor regression (Table 1, Fig. 8). Pilot experiments had already shown that this was the maximum concentration of Oleuropein that could be used, since higher concentrations were too bitter and the mice refused to drink. Oleuropein induced complete tumor regression in 10/11 mice and partial regression in one animal. It seems that Oleuropein had a similar effect on mice bearing single (mouse B) or multiple (mouse A) tumors but the increased tumor load may have slightly delayed the time to complete regression (9 vs. 12 days). The complete regression of such large tumors (>2 cm in diameter) within 12 days was surprising and reproducible (Table 1). Interestingly, after the tumors regressed, the region where the tumor had been located was sunken, with excess skin folds and a dark underlying area (Fig. 8). Within a week, the area became normal (not shown). After tumors regressed, Oleuropein treatment was discontinued and mice were returned to regular drinking water. All mice remained tumor-free for the remainder of their lifespan (Table 1). It is unknown whether this tumor-free state was due to the Oleuropein treatment or to the nature of the animal model itself. It is possible that tumor development was age-dependent. Because this mouse strain arose through a natural occurrence without human intervention, the exact mechanism by which the tumors develop is unclear. Because all untreated animals die within 2 weeks of tumor appearance, it was not possible to determine whether tumors would have continued to appear if the animal had survived.

In order to obtain samples for analysis, we resected incompletely regressed tumors on day 5 of Oleuropein treatment (not shown). We noticed that the Oleuropein-treated mice had tumors of a non-cohesive, crumbly consistency, unlike tumors from non-treated animals, which were more fibrous and solid.

Table 1
Experimental outcomes and survival for control and Oleuropein-treated mice

Mouse number	Ole treatment (9–12 days)	Experimental outcome	Survival (months)
1	None	Increased tumor	0
2	None	Increased tumor	0
3	None	Increased tumor	0
4	None	Increased tumor	0
5	None	Increased tumor	0
6	Oral (ad libitum)	Complete regression	18+
7	Oral (ad libitum)	Complete regression	14
8	Oral (ad libitum)	Complete regression	18+
9	Oral (ad libitum)	Complete regression	18+
10	Oral (ad libitum)	Complete regression	12
11	Oral (ad libitum)	Complete regression	18
12	Oral (ad libitum)	Complete regression	12
13	Oral (ad libitum)	Complete regression	15+
14	Oral (ad libitum)	Complete regression	14+
15	Oral (ad libitum)	Complete regression	14+
16	Oral (ad libitum)	Partial regression	3
17	Oral (ad libitum)	Early resection	N/A
18	IP injection	Early resection	N/A

Ole, Oleuropein. IP, intraperitoneal injection. "+" denotes the mouse was still alive at the time of manuscript submission.

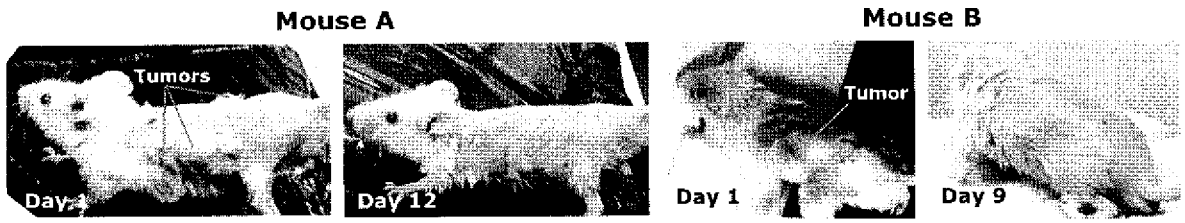

Fig. 8. Oleuropein induces tumor regression in vivo. Mice bearing spontaneous tumors were administered 1% Oleuropein in their drinking water. Some mice had multiple tumors (represented by mouse A), whereas others bore a single tumor mass (represented by mouse B). After 9–12 days of treatment, tumors had completely regressed.

We attempted to develop primary tumor cell lines from these Oleuropein-treated tumors without success. Therefore, it was necessary to shorten the treatment to 2 days. Since we were unsure whether the mice would drink enough in a 2-day period, we injected Oleuropein intraperitoneally. After 2 days, the tumor was removed and analyzed (Fig. 9). This tumor appeared heterogeneous (Fig. 9A, inset) and was still vascularized. Parts of the tumor, especially those near blood vessels, exhibited a crumbly consistency (Fig. 9B) containing clusters of rounded cells (Fig. 9C). The small fragments that separated from the main tumor mass during dissection were mostly composed of rounded cell clusters (Fig. 9D) and resembled the disrupted tumor spheroids we observed in the Matrigel invasion and tube-disruption assays (Figs. 4 and 5). Intriguingly, we also observed a crystalline accumulation in the crumbly parts of the tumor (Fig. 9D, arrowhead). These crystals were similar in shape and size to Oleuropein crystals in a saturated solution (not shown) but their identity remains undetermined.

Discussion

Since the 1800s, the bitter component in olives was used in humans against malaria-induced fevers. Later studies showed that it also possessed anti-microbial, anti-viral, and anti-fungal activities [24,25]. In 1960, Panizzi and Oriente [26] succeeded in isolating Oleuropein from the olive bitter fraction. Its chemical structure was definitively elucidated in 1970 by Inouye et al. [27] using Oleuropein purified from the Japanese privet tree *Ligustrum japonicum*. Purified Oleuropein and its metabolite elenolic acid also possessed anti-microbial, anti-viral, and anti-fungal properties (reviewed in [28]). Further experimentation showed that it was a powerful antioxidant [29,30] as well as a hypotensive [31,32] and hypoglycemic agent [32,33]. It also exerted protective effects against heart disease and had immunoregulatory actions [19,34,35].

Some studies were undertaken to determine the toxicity of Oleuropein and its two main metabolites (hydroxytyrosol and elenolic acid); all were found to be completely non-toxic in several animal species

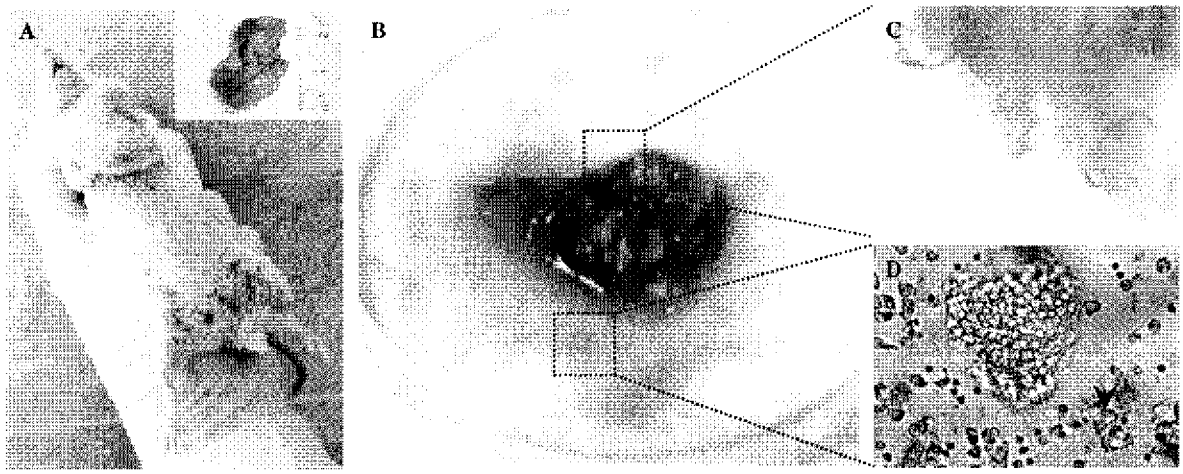

Fig. 9. Oleuropein induces tumor cell rounding in vivo. One mouse (A) was given a 500 ml intraperitoneal injection of a 10% Oleuropein solution in Hanks' phosphate-buffered saline every 12 h for 2 days. The tumor (A–D) was resected and measured on day 3 (A, inset). The tumor was heterogeneous and had crumbly regions (B) containing clusters of rounded cells (C,D) and crystals (arrowhead). Magnification: (B), 10×; (C) and (D), 100×.

[31,36,37]. In acute toxicity studies for Oleuropein, no lethality or adverse effects were observed in mice even when it was administered at doses as high as 1000 mg/kg, thus an LD_{50} could not be determined [31]. Also, we have previously shown that injection of Oleuropein into fertilized chicken eggs did not interfere with normal embryonic development [20]. Because developing embryos are extremely sensitive to toxicity, these results provide even stronger support for the safety of Oleuropein. Importantly, the breakdown products of Oleuropein, hydroxytyrosol and elenolic acid, exhibited no toxicity even at doses as high as 2000 mg/kg of body weight [36,37]. Even though several human studies have been conducted with olive extract or its polyphenolics showing no adverse effects (reviewed in [29,34,38–40]), more systematic efforts are needed to examine the safety of Oleuropein in humans.

Given that Oleuropein is a non-toxic compound with numerous beneficial properties, we wondered if it had effects on cancer cells. In this paper, we demonstrate its direct anti-tumor activities. We show that Oleuropein inhibits cell growth, motility, and invasiveness. We also show that it induces cell rounding, a phenomenon that has been linked to the disruption of the actin cytoskeleton [41]. We indeed have shown that Oleuropein disrupts the actin cytoskeleton of living cells within 2 h as well as directly disrupting purified actin filaments in a cell-free assay (Fig. 7). In cells, this effect was partially counteracted by the concomitant addition of glucose to the cultures. Given the fact that removal of Oleuropein's glucose moiety with β-glucosidase decreases its anti-proliferative activity (Fig. 2), it is possible that glucose transporters (GLUTs) may facilitate the diffusion of Oleuropein into the cell. There are currently 12 GLUT proteins with various glucose affinities and tissue-specific distributions [42]. It has been reported that human malignancies are characterized by elevated glucose uptake and utilization based on enhanced expression of multiple GLUT isoforms [43]. For example, GLUT1 and/or GLUT3 mRNA and/or protein was increased in cancers of the cervix, thyroid, prostate, breast, and colon [43–47]. Thus, it is likely that cancer cells that overexpress specific GLUT proteins would be more likely to uptake Oleuropein. This might explain the differential sensitivity to Oleuropein of the various cancer cell lines we tested (leukemia > melanoma > colon and breast > kidney) shown in Fig. 1. The fact that most normal cells have low or no expression of certain GLUTs [46] may explain the reversibility of cell rounding in normal but not in cancer cells, after Oleuropein treatment (Fig. 6). In spite of the above, it is likely that Oleuropein also enters the cell by other routes. This is supported by the fact that competition with excess glucose as well as β-glucosidase treatment did not completely abolish the biological activity of Oleuropein (Figs. 2, 5, and 7).

Interestingly, the Oleuropein-induced cell rounding observed in our in vitro experiments was also detected in vivo. In our animal studies, tumor regression occurred quite rapidly, with large (>2 cm diameter) tumors completely disappearing within 9–12 days. This is quite unique among chemotherapeutic agents, since most of them do not induce complete regression in such a short time. This rapid effect of Oleuropein on tumors could be explained by its almost immediate effect on the cytoskeleton, which we observed in vitro. To determine whether cell rounding also occurred in vivo, we examined partially regressed tumors. Even a short, 2-day treatment with Oleuropein was sufficient to induce cell rounding within the tumor itself without having obvious effects on the vasculature (Fig. 9). Our data suggest that the principal anti-tumor mechanism of Oleuropein in vascularized tumors involved the direct disruption of tumor cells.

To combat cancer, medicine has relied on toxic compounds [48]. Most therapies do not discriminate between normal and cancer cells, leading to toxicity and unwanted side effects. In recent years, major efforts have focused on identifying and testing anti-angiogenic compounds as cancer therapeutics. Although this anti-cancer approach appeared very promising when first proposed, it has not been as successful as envisioned [49]. It appears that anti-angiogenic therapies would be more successful if used in chemoprevention rather than on established tumors. Recent developments show that powerful survival mechanisms are triggered in cancer cells under hypoxic conditions [49]. This may explain the limited success of most anti-angiogenic therapies, in which the surviving patients experience more aggressive tumor growth after the initial treatment [50]. These arguments are better understood in the context of the tumor microenvironment. In the nascent tumor containing less than 300 cells, there is minimal genetic variability. At this stage, a tumor is more vulnerable to drugs because all the cells are equally susceptible, due to the clonal nature of the tumor. It is at this point that anti-angiogenic therapies (chemo-preventive) would be most effective by preventing further growth of the tumor. Unfortunately, existing technology is not yet able to detect a clump of 300 cancer cells and patients go untreated. As the tumors become vascularized, the number of cells increases exponentially and tumor cell genetic variability becomes extensive. It is at this later stage that current technology can detect tumors and cancer therapies are thus initiated. If an anti-angiogenic therapy is given when the tumor is already highly vascularized, a selection process ensues in the tumor leading to the growth of hypoxia-resistant cells, which can survive and proliferate in this new environment [49,51]. Because Oleuropein quickly targets tumor cells prior to its targeting of tumor vasculature, a selection process driven by hypoxia may not take place.

We propose that Oleuropein represents a new class of anti-cancer compounds, which targets multiple steps in cancer progression. As an antioxidant, it may protect cells from incurring genetic damage leading to oncogenesis. As an anti-angiogenic agent, it can prevent tumor progression. Finally by directly inhibiting cancer cells, it can lead to tumor regression. The unique combination of these properties in a single molecule should elevate Oleuropein from a dietary component into an active cancer drug worthy of human studies.

Acknowledgment

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Oleuropein, a non-toxic olive iridoid, is an anti-tumor agent and cytoskeleton disruptor.

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Oleuropein, a non-toxic secoiridoid derived from the olive tree, is a powerful antioxidant and anti-angiogenic agent. Here, we show it to be a potent anti-cancer compound, directly disrupting actin filaments in cells and in a cell-free assay. Oleuropein inhibited the proliferation and migration of advanced-grade tumor cell lines in a dose-responsive manner. In a novel tube-disruption assay, Oleuropein irreversibly rounded cancer cells, preventing their replication, motility, and invasiveness; these effects were reversible in normal cells. When administered orally to mice that developed spontaneous tumors, Oleuropein completely regressed tumors in 9-12 days. When tumors were resected prior to complete regression, they lacked cohesiveness and had a crumbly consistency. No viable cells could be recovered from these tumors. These observations elevate Oleuropein from a non-toxic antioxidant into a potent anti-tumor agent with direct effects against tumor cells. Our data may also explain the cancer-protective effects of the olive-rich Mediterranean diet.
PMID: 16024000 [PubMed - indexed for MEDLINE]

EXHIBIT 9

Up-regulation of glucose transporter 1 (GLUT1) in human cancer

Cancer Res. 1996 Mar 1;56(5):1164-7

Wide expression of the human erythrocyte glucose transporter Glut1 in human cancers.

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Glucose uptake has been found to be increased in cancer cells. Previous work has shown increased expression of the human erythrocyte glucose transporter (Glut1) mRNA in some human cancers, indicating that Glut1 may play a significant role in glucose uptake by these tumors. The distribution of Glut1 protein in normal and malignant human tissues is still largely unknown. Using immunohistochemistry, we found that Glut1 is largely undetectable in normal epithelial tissues and benign epithelial tumors but is expressed in a significant proportion of a variety of human carcinomas. We hypothesize that Glut1 expression by human carcinomas indicates an increased glucose uptake, and probably increased utilization of energy, which may correlate with an aggressive behavior. The biological significance of Glut1 expression needs to be determined.

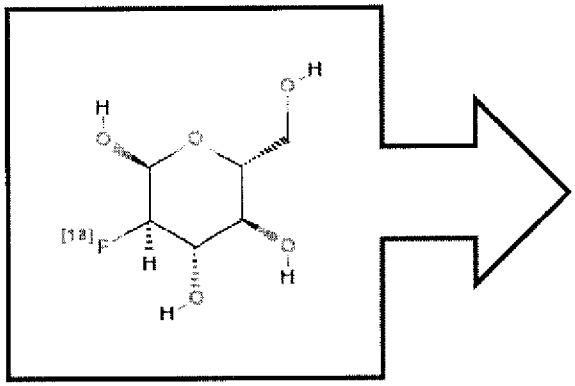
PMID: 8640778 [PubMed - indexed for MEDLINE]

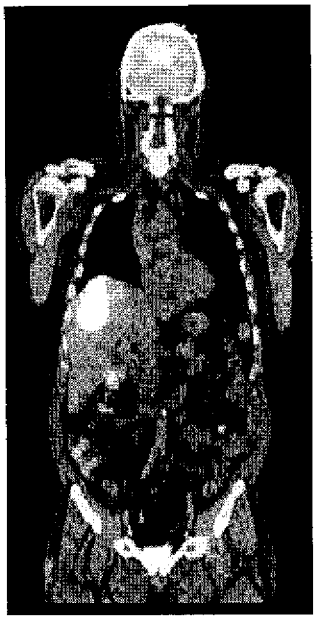
EXHIBIT 10

Global use of FDG-PET scan in cancer

PET-CT

Fluorodeoxyglucose F18 (FDG)





J Nucl Med. 2005 Jun;46(6):983-95

Use of PET for monitoring cancer therapy and for predicting outcome.

Weber WA.

Department of Molecular and Medical Pharmacology, Ahmanson Biological Imaging Center, UCLA David Geffen School of Medicine, 10833 Le Conte Avenue, Los Angeles, CA 90095-6942, USA. wweber@mednet.ucla.edu

PET with the glucose analog (18)F-FDG is increasingly used to monitor tumor response in patients undergoing chemotherapy and chemoradiotherapy. Numerous studies have shown that (18)F-FDG PET is an accurate test for differentiating residual viable tumor tissue from therapy-induced fibrosis. Furthermore, quantitative assessment of therapy-induced changes in tumor (18)F-FDG uptake may allow the prediction of tumor response and patient outcome very early in the course of therapy. Treatment may be adjusted according to the chemosensitivity and radiosensitivity of the tumor tissue in an individual patient. Thus, (18)F-FDG PET has an enormous potential to reduce the side effects and costs of ineffective therapy. This review focuses on the practical aspects of (18)F-FDG PET for treatment monitoring and on how to perform a quantitative assessment of tumor (18)F-FDG uptake in clinical studies.

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Nucl Med Biol. 1996 Aug;23(6):717-35.

PET and [18F]-FDG in oncology: a clinical update.

Conti PS, Lilien DL, Hawley K, Keppler J, Grafton ST, Bading JR.

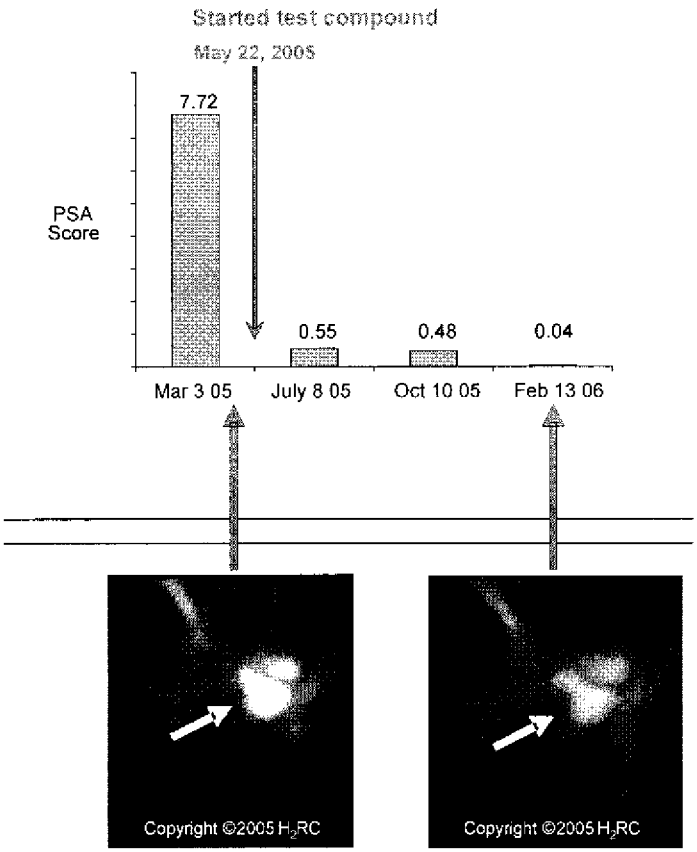
Department of Radiology, University of Southern California, Los Angeles 90033, USA.

Positron emission tomography (PET) has become a very useful adjunct to anatomic imaging techniques, adding unique information to the characterization of disease. The whole-body PET FDG technique developed over the last few years has surpassed most expectations with respect to its utility in clinical oncology. The large spectrum of neoplasms that now can be studied with this approach makes it an essential clinical imaging tool in diagnosis and management for many patients with cancer. The metabolic information provided by this technique is complementary to results from standard clinical and morphological examinations. It may be anticipated that through application of the multi-modality imaging approach, significant advances in medical care will come.

EXHIBIT 11

The efficacy of the test compound in humans

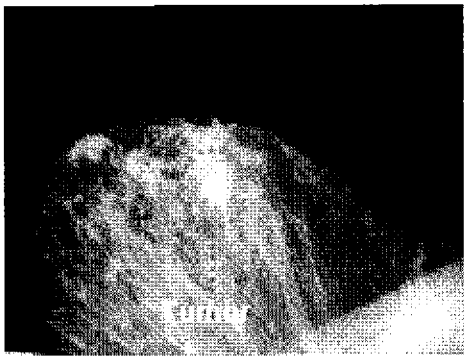
53 y.o. male with prostatic adenocarcinoma, extracapsular disease, confirmed pathologically. Patient given casodex and lupron without effect on tumor or PSA. Subsequent bone-scan also showed a **metastasis** on the right symphysis pubis. The compound was administered orally for 8+ months without reported side effects. By week 6 PSA dropped (graph). By the 8th month, new bone-scan showed disappearance of the pubic metastasis and PSA score remained low.



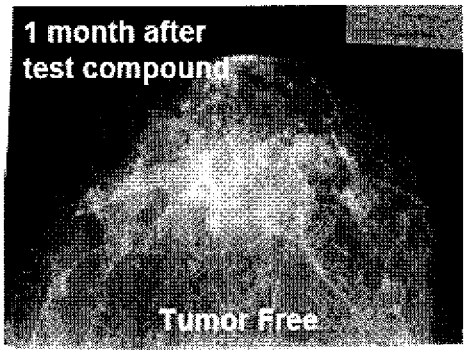
Bone scan 4/13/2005

Bone scan 2/13/2006

63 year-old. female presented with a large, palpable mass in her left breast. Scans and mammogram determined that her tumor measured 5 x 6 x 7.5 cm and that it had metastasized to multiple lymph nodes. Biopsy confirmed the diagnosis of invasive carcinoma, PR+++ and HR+++. Doctors advised a total mastectomy with removal of lymph nodes but she started taking the test compound. After one month of treatment, ultrasound and mammogram showed no tumor in the breast or lymph nodes.



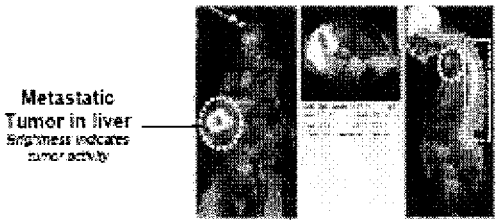
Mammogram
Sep 12, 2006
Left Breast



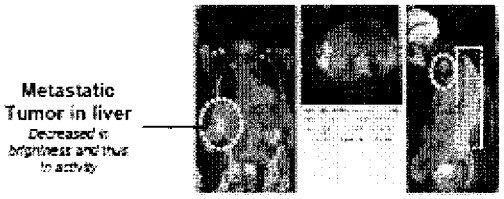
Mammogram
Dec 23, 2006
Left Breast

62 y.o. male, extremely obese (344 lbs) with multiple health problems: kidney disorders including calcifications, scoliosis and other spinal malformations with pain, including inability to straighten neck due to extreme bone degeneration, severe fatigue, recurrent infections (chronic bacterial ostitis), heavy metal accumulations, hypothalamic dysfunction, abdominal hernia. He has had several hernia operations. Family history of cancer (father died of colon cancer; brother died of lung cancer. Diagnosed with colon adenocarcinoma in April 2006 and underwent colectomy the same month (he uses a colostomy bag). At the same time, studies indicated several metastatic lesions to the liver and various lymph nodes. He underwent chemotherapy (FOLFOX-4 protocol) and experienced severe symptoms including infected port-a-cath (needed surgery to replace), heart damage and peripheral neuropathy that made it impossible to walk without a walker. Slight response to chemotherapy as assessed by decreased CEA values (3 point decrease over length of treatment). As soon as chemotherapy ended, CEA values increased (more than 5 points in 6 months). PET/CT scan showed marked growth and increased activity in the five hepatic masses. Further standard chemotherapy was not recommended due to existing health conditions. Started test compound on 01-17-2007. After 4 months the tumor has shrunk and decreased in activity. Patient is still on the test compound and is doing very well.

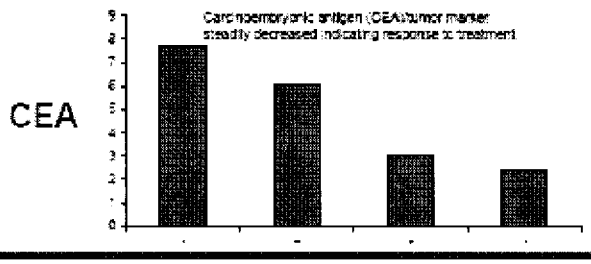
Patient started treatment on 01-17-2007



^{18}FDG PET/CT
01-16-2007



^{18}FDG PET/CT
04-07-2007



A 16 year-old male presenting with a 10x14cm osteosarcoma on the dorsal area of the knee with tibial involvement. Prior treatment included several chemotherapy cycles for approximately 9 months without success. The mass was deemed inoperable due to the involvement of neighboring structures such as blood vessels, muscles and nerves. Test compound was orally administered for 8 weeks and no side effects were reported. Within 2 weeks, the tumor started to lose its hard consistency upon palpation and the area showed reduced inflammation and pain. Within 4 weeks, the tumor was significantly softer. At 6 weeks, the tumor detached from the tibia and surrounding structures, and was left hanging within the attached skin. By 8 weeks, the oncologist in charge of the case assessed that the tumor was >90% dead as assessed by the multiple MRI scans and biopsy.

A 56 year-old female with breast carcinoma. Initial mastectomy followed by chemotherapy. Recurrence within 2 years to axillary lymph node, followed by several rounds of chemotheraphy, achieving tumor disappearance. Recurrence within 9 months to axillary lymph node followed by chemotheraphy and radiation without significant success. Three months after, a new tumor appeared in the supraclavicular Virchow's node which was unresponsive to chemotherapy and deemed unresectable due to involvement of adjacent structures. Test compound was administered orally for 12+ weeks; no side effects were reported. At week 12, based on scan results, oncologist conducted a surgical resection of the Virchow's node mass. Tumor mass consisted entirely of dead cells according to pathology reports.

84 year-old male with multiple pancreatic tumors and abdominal metastases at presentation. Due to age and advanced stage at moment of diagnosis, no intervention was attempted and patient was only given morphine for pain management. Test compound was orally administered for 7 weeks and no side effects were reported. At week 7, MRI showed all tumors had disappeared. Patient was functioning normally, and even traveling internationally 4 weeks after ending treatment. No recurrence has been determined to this date (18 months later).

35 year-old male who had a 1.8 X 1.8 X 1.5 cm tumor in left temporal lobe of the brain. The tumor was completely removed surgically; pathology reports proved it to be glioblastoma multiforme (Astrocytoma Grade IV) with extensive vascularization and aggressive features. Patient received 30 sessions of Radiation Therapy concurrently with 140 mg of Temodal daily for a period of 41 days. MRI performed 2 months later showed the appearance and growth of two tumors in the same brain region, the largest measuring 1.6 X 1.2 cm in spite of the treatment. Patient then started taking test compound daily, together with low doses of Temodal only 5 days per month. MRI taken 2 ½ months later showed that one tumor had disappeared and the other shrunk 70%. Patient continued taking test compound daily, together with Temodal as previously described. MRI taken 3 ½ months after showed no evidence of tumors.

A 39 year old female presented with a 3.4cm mass in left breast. Three needle core biopsies confirmed the presence of a highly aggressive Grade III infiltrating ductal carcinoma extending into neighboring lymph nodes. Immunocytochemistry analysis: (-) HER-2/neu; (-) ER; (-) PR; Genetic analysis showed mutation in BRCA2 gene. Patient received radiation therapy and chemotherapy (adriamycin, 5-FU, taxol, cyclophosphamide) without responding. Surgery was conducted to remove breast and 18 lymph nodes with 11 being (+) for cancer. Patient was given taxotere for maintenance. Within 1 year, a suspicious lesion appeared in the other breast; thus, patient underwent a prophylactic mastectomy followed by further chemotheraphy. Based on her medical history and family history (mother, sister died of cancer), she was given 85% chance that the cancer would recur within 6 months. Patient started test compound (continuous maintenance dose); regular ultrasounds, CT scans, bone scans, MRIs, and blood tests for tumor markers (CEA and CA27.29) show that she has remained cancer-free for 4+ years.